Supplementary Material

Effects of Y361-auto-phosphorylation on structural plasticity of the HIPK2 kinase domain.

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Expression, purification of HIPK2^{WT}, HIPK2^{Y361F} and HIPK2^{K228A} KDom.

Recombinant HIPK2 KDom wild type and mutants were overexpressed *E. coli* BL(21). Cells were transformed and grown at 303 K in LB medium, containing 100 μg/ml ampicillin, up to OD600 ≈ 0.8 before adding 0.3 mM isopropyl-d-thiogalactopyranoside (IPTG) and incubating for 16 hours at 289 K. The harvested pellet was resuspended in 50 mM Tris·HCl, 300 mM NaCl, 2 mM DTT, 50 mM arginine, 50 mM glutamate, 5% glycerol, pH 8 (Lysis Buffer) and lysed by addition of a mixture of deoxyribonuclease, ribonuclease, lysozyme (Sigma Aldrich, St. Louise, Missouri, USA), and protease inhibitors (cOmplete, EDTA-free, Roche, Basel, Switzerland), followed by sonication. After centrifugation, the soluble fraction of the lysate was loaded onto a 5 ml Glutathione Sepharose Fast Flow column equilibrated with Lysis Buffer and eluted using 2.5 mM glutathione. In order to cleave the GST tag, the sample was incubated overnight at 277 K with 2 units of PreScission protease (Ge Healthcare, UK) per 100 μg of protein. Reverse GST-affinity chromatography was performed to remove uncleaved protein and GST. To check sample homogeneity and degree of monodispersion, protein sample was loaded into a BioFox 17/1200 SEC (Knauer -Berlin, Ge) gel permeation column, and eluted in isocratic mode in 50 mM Tris·HCl, 300 mM NaCl, 2 mM dithiothreitol (DTT), 5% Glycerol, pH 8.0. HIPK2^{WT}, HIPK2^{Y361F}, and HIPK2^{K228A} KDom purification results in a protein of 45.6 kDa (75.5 kDa with GST-tag). Purified samples were concentrated and stored at 193 K. Quality and quantity of purified proteins were evaluated by SDS-PAGE (See Supplementary Figure S1) and UV/visible spectra using the theoretical ε_{280nm}= 48820 M^{-1} cm^{-1}.

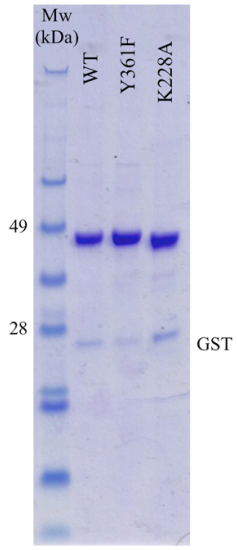


Figure S1. Purity assessment of HIPK2^{WT}, HIPK2^{Y361F} and HIPK2^{K228A} KDom. Purified HIPK2^{WT} (WT), HIPK2^{Y361F} (Y361F) and HIPK2^{K228A} (K228A) KDom (165-564) analysed on SDS-PAGE denaturing gel, stained by Coomassie blue. Molecular Weight (Mw) Marker: SeeBlue Prestained Standard (Thermo Fisher Scientific, USA). The lack of phosphorylation on K228A mutant can be appreciated by the slight decrease in the apparent molecular weight with respect to WT and Y361F.

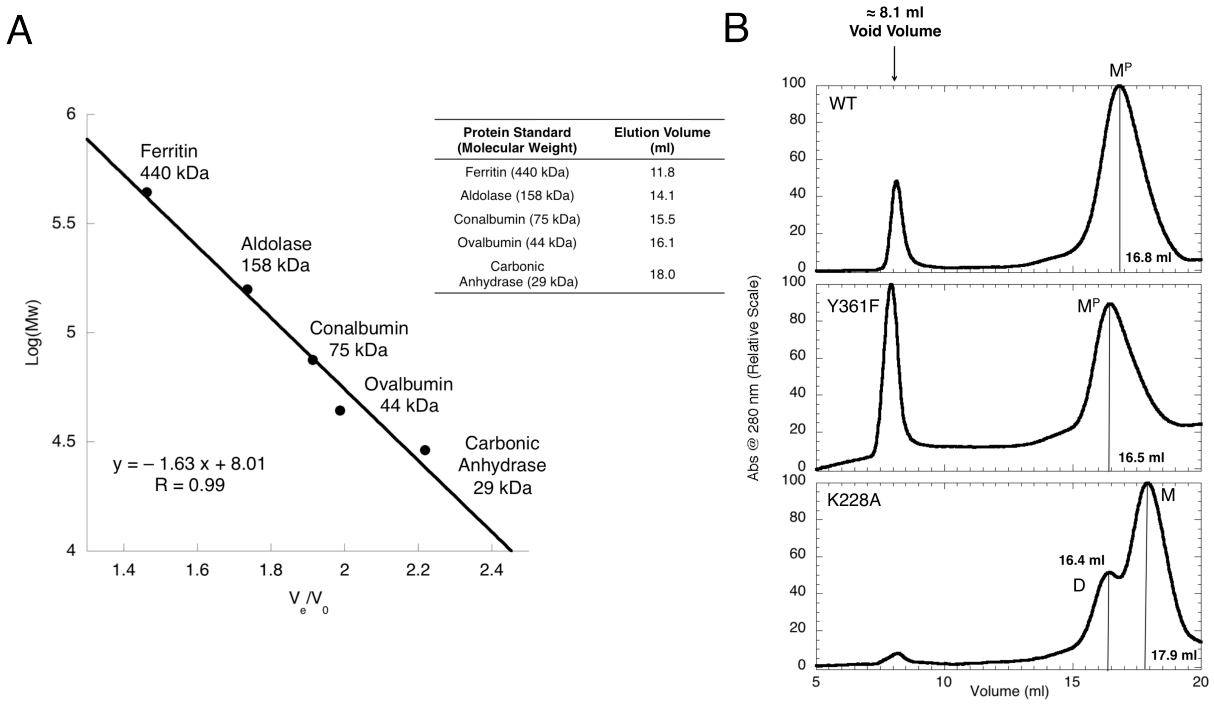


Figure S2. (A) Column Calibration. Biofox 17/1200 SEC column was calibrated at room temperature in 50 mM Tris·HCl, 0.3 M NaCl, 5 mM TCEP, 5% Glycerol, pH 8. The decimal logarithm of the molecular weight standards is reported as a function of the ratio between the measured elution volume (V_e) determined by following the absorbance signal at λ= 280 nm and the void volume ($V_0 \approx$ 8.1 ml). The measured elution volume for each molecular weight standard is listed in table. Analysis of chromatograms (shown in Figure 1B) showed that HIPK2^{WT} KDom and mutants elute predominantly as monomers (Mw ~ 45.6 kDa) with a main peak centred in a range of volumes typical of a 40 kDa protein (Ovalbumine 44 kDa, elution volume ≈ 16.3 ml). HIPK2^{Y361F} displays a higher tendency to form high molecular weight oligomers, showing also a peak in the void volume of the column (~ 8.4 ml) and peaks at volumes that may be ascribed to different oligomeric species (Figure 1A), at 11.4 ml (typical of a 440 kDa protein, *i.e.* Ferritin) and at 14.2 ml (typical of a 158 kDa protein, *i.e.* Aldolase). M= monomer, M^P= monomer with phosphorylations, D= dimer. (**B) HIPK2^{WT}, HIPK2^{Y361F}, HIPK2^{K228A} chromatographic profiles after 1 hour of incubation at 310 K.** SEC of HIPK2^{WT} (WT), HIPK2^{Y361F} (Y361F), HIPK2^{K228A} (K228A) after 1

hour of incubation at 310 K was performed in isocratic mode in 50 mM Tris · HCl, 0.3 M NaCl, 5 mM TCEP, 5% Glycerol, pH 8 using a Biofox 17/1200 SEC column. Sample elution was monitored following the absorbance signal at λ= 280 nm at room temperature. If compared with Figure 1B the disappearance of the high molecular weight oligomeric species is observed, thus indicating that these species aggregate upon thermal incubation at 310 K. Consistently the peak eluting at the void volume of the column is increased.

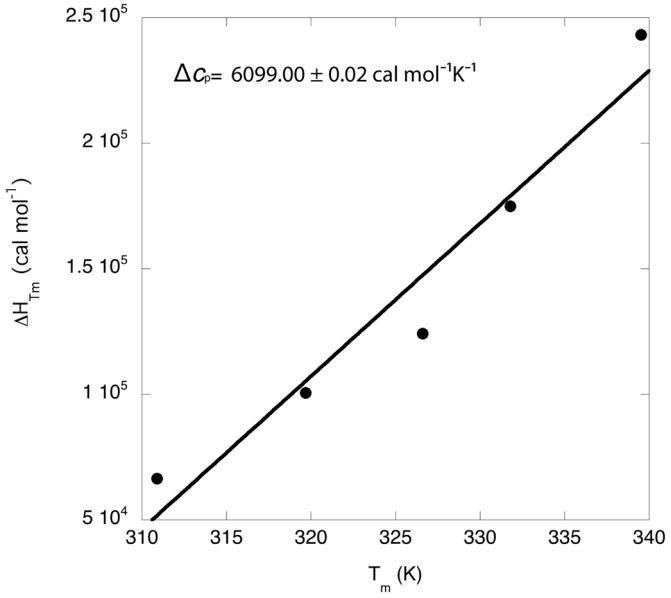


Figure S3. Experimental determination of Δc_p. Dependence of ΔH_{Tm} on T_m measured at different pH values ranging from pH 5.0 to 10.0. Solid line is the best fit to a linear equation, returning a slope Δc_p = 6099.00 ± 0.02 cal $mol^{-1}K^{-1}$. Thermal denaturation was followed at 222 nm with heating from 293 to 363 K at a rate of 2 K min^{-1}. Buffers used were 50 mM 2-(Cyclohexylamino)ethanesulfonic acid (CHES) pH 10.0, potassium phosphate pH 8.0, 7.0 and 6.0, Tris·HCl pH 7.5, sodium acetate pH 5.0, 150 mM NaCl (28). At temperature over 350 K a second transition was observed due to protein aggregation and precipitation. However, measuring the unfolding parameters for $HIPK2^{WT}$ KDom at different rates of heating returned the same apparent unfolding parameters, thus showing that kinetics of aggregation does not affect the results (30). Thermal denaturation was analysed by fitting the transition region data (293-350 K) to a two-state model (27).

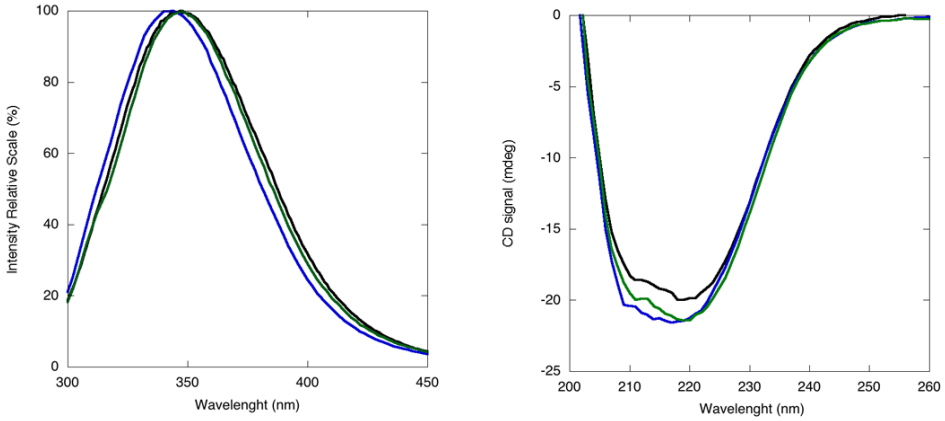


Figure S4. Fluorescence and CD spectra of HIPK2^{WT}, HIPK2^{Y361F} and HIPK2^{K228A}. Fluorescence emission spectra (left panel) and Far-UV CD spectra (right panel) of 6 μM HIPK2 KDom wild type (black), HIPK2^{Y351F} (blue) and HIPK2^{K228A} (green) were recorded respectively in 50 mM Hepes and 200 mM NaCl, pH 7.5 and 50 mM potassium phosphate and 150 mM NaCl, pH 8.0 at 293 K. Fluorescence emission spectra were recorded between 300 and 400 nm with an excitation wavelength of 280 nm using a Fluoromax spectrofluorimeter (Jobin Yvon, Edison, NJ, USA) in a 1 cm quartz cuvette. Far-UV CD spectra of HIPK2^{WT} KDom and mutants were recorded between 260 and 200 nm using a Jasco spectropolarimeter.

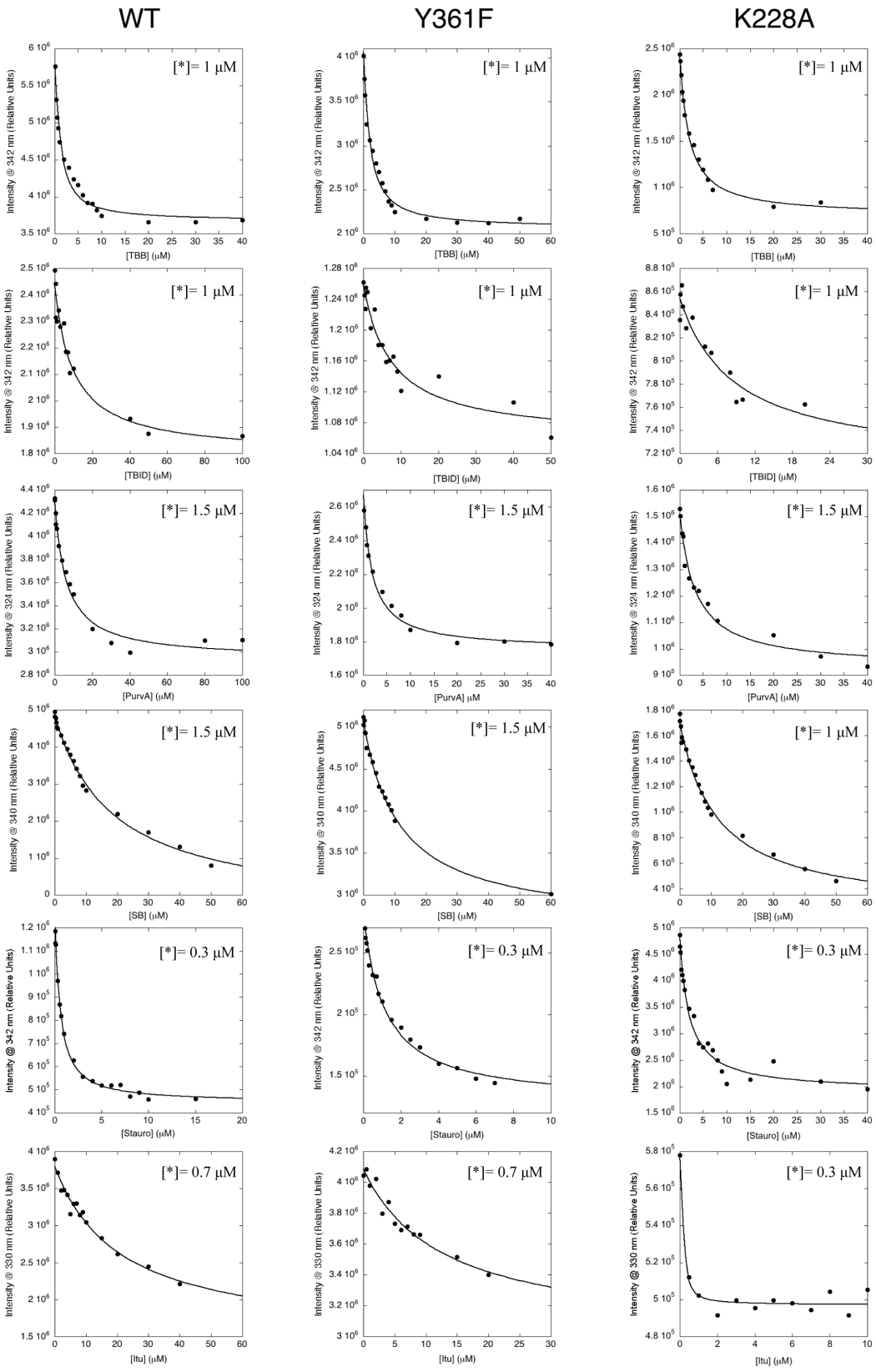


Figure S5. Binding of ATP-competitors to HIPK2^{WT} and mutants. Equilibrium binding titrations of HIPK2^{WT} (WT), HIPK2^{Y361F} (Y361F) and HIPK2^{K228A} (K228A) with 4,5,6,7-

tetrabromobenzimidazole (TBB), 1H-imidazol-2-yl-4,5,6,7-tetrabromoisoindoline-1,3-dione (TBID), Purvalanol A (PurvA), SB203580 (SB), Staurosporine (Stauro), 5-Iodotubercidin (Itu) monitored by the change in intrinsic fluorescence of the protein followed at 324 nm for PurvA, 330 nm for Itu, 340 nm for SB and 342 nm for TBB, TBID and Stauro, because of the different optical properties of the compounds. The experiments were performed at 293 K in 50 mM Hepes and 200 mM NaCl, pH 7.5, at a constant concentration of protein ([*]). The final concentration of DMSO was kept below 1%.

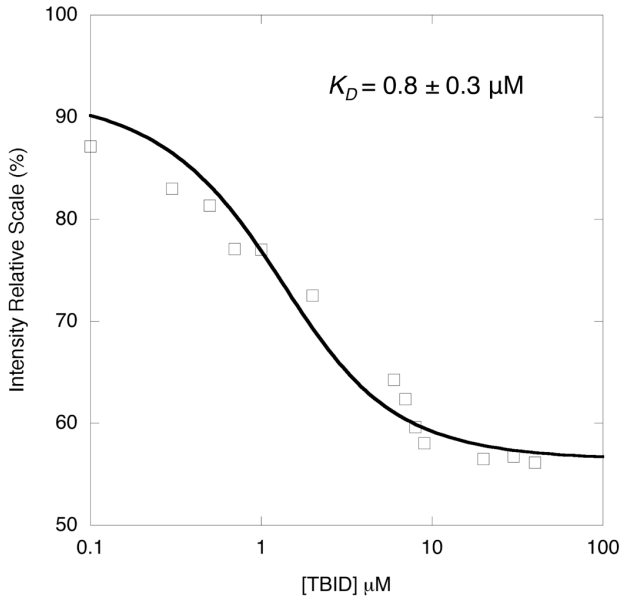
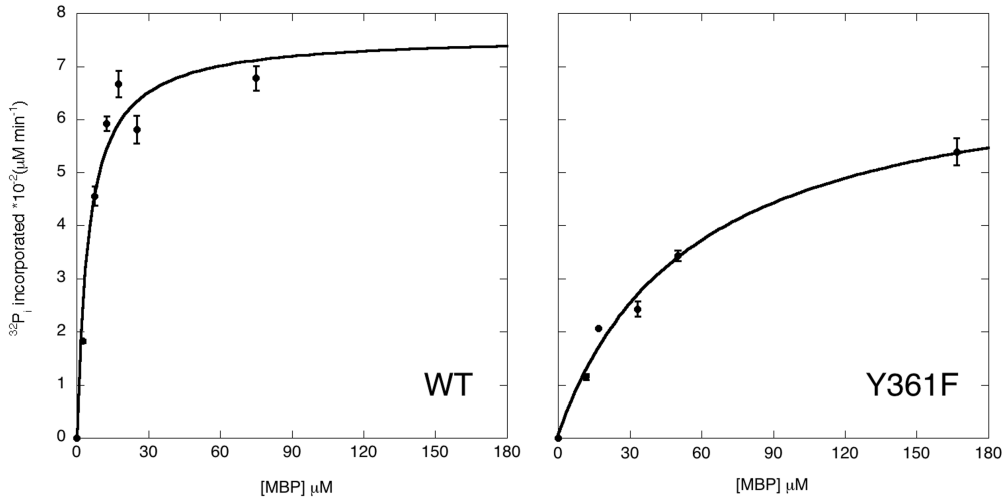


Figure S6. TBID equilibrium binding experiment at 310 K. Since the difference of one order of magnitude between the K_D estimated at 293 K in this work (8.0 μM, Table 1) and the IC_{50} previously determined for TBID by Cozza *et al*. at 310 K (IC_{50}= 0.33 μM) could be due to the difference in temperature at which experiments were performed, the equilibrium binding experiment for this compound was repeated at 310 K following the intrinsic fluorescence quenching of murine $HIPK2^{WT}$ that occurs upon TBID binding in 50 mM Hepes and 200 mM NaCl, pH 7.5, using a constant concentration of protein (1 μM), varying inhibitor concentration. The relative fluorescence intensities at 342 nm, corrected for the inner filter effect, are plotted as function of TBID concentration on a logarithmic scale and fitted to quadratic equation, returning a K_D= 0.8 ± 0.3 μM, comparable to what previously reported by Cozza *et al* (32).



	K_M (μM)	V_{max} (μM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})
HIPK2^{WT}	5.1 ± 0.9	0.076 ± 0.003	0.173 ± 0.003	0.034 ± 0.006
HIPK2^{Y361F}	54 ± 7	0.071 ± 0.004	0.081 ± 0.005	0.0015 ± 0.0002

Figure S7. Kinetic analysis on the kinase activity on MBP. The phosphorylation of MBP by HIPK2^{WT} (WT, 0.4 μM) and HIPK2^{Y361F} (Y361F, 0.8 μM) was carried out in kinase buffer (20 mM Hepes pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$), varying MBP concentration between 0-170 μM and using [γ-^{32}P]-ATP (6*10^5-8*10^5 cpm nmol^{-1}) at 303 K. After a preincubation of 10 minutes at 303 K, reactions were initiated with the addition of [γ-^{32}P]-ATP in a total reaction volume of 10-15 μl. The reactions were terminated after 20 min by adding 2-3 μl of 0.5 M phosphoric acid on ice. At that time ^{32}P incorporation was linear with respect to time. The entire volume of the reaction was spotted onto a phosphocellulose filter disk and was washed 4 times with 75 mM phosphoric acid. The filter disks were counted on the ^{32}P channel in liquid scintillant. The total amount of phosphate incorporated into the substrate was then determined by considering the specific activity (cpm) of the reaction mixture, and the background retention of unreacted [γ-^{32}P]-ATP and HIPK2 autophosphorylation in the absence of MBP. Each condition has been assayed at least in duplicate. Data were fitted using the Kaleidagraph software package and Prism (Graphpad) by following the classical hyperbolic equation of Michaelis−Menten.

Table S1. Sedimentation velocity experiments on HIPK2 wild type and mutants (Y361F and K228A).

Sample Name	$S_{20,W}$	$Mw_{experimental}$ (kDa)
$HIPK2^{WT}$	3.6	45
$HIPK2^{Y361F}$	3.9	45
$HIPK2^{K228A}$	5.4; 3.8	85; 45

Sedimentation velocity of $HIPK2^{K228A}$ after size exclusion chromatography. The sample was loaded into a BioFox 17/1200 SEC gel permeation column, and eluting peaks have been isolated before the ultracentrifugation (UC) analysis. The species eluting at 17.9 ml shows one symmetric peak corresponding to a sedimentation coefficient at 20°C ($S_{20,W}$) of 3.8 S ($Mw_{experimental}$~ 45 kDa), matching the molecular weight of the monomer (45.6 kDa), while the one eluting at 16.4 ml shows a peak corresponding to a $S_{20,W}$ of 5.4 S ($Mw_{experimental}$~ 85 kDa), that is compatible with the molecular weight of the dimer (~ 90 kDa).

The table also reports sedimentation velocity coefficients of $HIPK2^{WT}$ and $HIPK2^{Y361F}$. Samples were loaded into a BioFox 17/1200 SEC gel permeation column and eluting peaks have been isolated before the UC analysis. The sedimentation profiles of the peak eluting at 16.9 ml of HIPK2 wild type and the peak eluting at 16.6 ml of $HIPK2^{Y361F}$ show one symmetric peak corresponding to a $S_{20,W}$ of 3.6 S and 3.9 S respectively, in full agreement with the molecular weight of the monomer. Data were recorded in a Beckman Optima XL-1 instrument equipped with absorbance optics. Protein samples were measured in the presence of 50 mM Tris·HCl, 300 mM NaCl, 5 mM TCEP, 5% glycerol, pH 7.5 at 20 °C. Radial absorbance scans were obtained in a continuous scan mode at 280 nm at a spacing of 0.003 cm. Sedimentation coefficients were calculated using the program Sedfit (provided by P. Schuck, National Institutes of Health) and were reduced to water and 20°C ($S_{20,W}$) according to standard procedures.